1.0 Purpose

The purpose of this procedure is to define the method for the quantitation and/or identification of berberine and/or dihydroberberine in raw materials and finished product dietary supplements using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of berberine and/or dihydroberberine in raw materials and finished products. Berberine is a good chromophore and was measured at 340, other wavelengths can be used to maximize signal to noise.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to ensure that this procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.2 **ACN –** Acetonitrile

4.3 **MeOH** – Methanol

4.4 **DMSO** – Dimethylsulfoxide

4.5 **H_2O_2** – Hydrogen Peroxide, 30%

4.6 **CofA** – Certificate of Analysis

4.7 **H_2O** – Water

4.8 **Berberine Chloride** – 5,6-Dihydro-9,10-dimethoxybenzo[*g*]-1,3-benzodioxolo[5,6-a]quinolizinium

4.9 **Dihydroberberine** – 9,10-Dimethoxy-6,8-dihydro-5H-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinoline

5.0 References

5.1 MV-LAB-18-178, Protocol, Berberine Determination using HPLC with UV/VIS Spectroscopy.

5.2 PRTCL-24-0030, Protocol, Validation of an Analytical Method for the Determination of Dihydroberberine by HPLC-UV

5.3 H. A. Weber , M. K. Zart , S. L. Ferguson , J. G. Greaves , A. P. Clark , R. K. Harris , D. Overstreet & C. Smith (2001) SEPARATION AND QUANTITATION OF ISOQUINOLINE ALKALOIDS OCCURRING IN GOLDENSEAL, Journal of Liquid Chromatography & Related Technologies, 24:1, 87-95, DOI: 10.1081/JLC-100000329

6.0 Supplies

6.1 Chemicals: All reagents are ACS grade or better.

6.1.1 H_2O ($\geq$ 18.2 MΩ·cm)

6.1.2 ACN

6.1.3 MeOH

6.1.4 DMSO

6.1.5 Ammonium acetate

6.1.6 H_2O_2

6.1.7 Berberine chloride reference standard

6.1.8 Dihydroberberine reference standard

6.2 Glassware **(Use Red Glassware When Conducting Assay of Dihydroberberine)**

6.2.1 HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.2 Volumetric Pipets

6.2.3 Mobile Phase Containers

6.2.4 50mL Volumetric Flasks

6.2.5 100mL Volumetric Flasks

6.3 Disposables

6.3.1 10mL Pipette Tips

6.3.2 200µL, 500µL & 1000µL Pipette Tips

6.3.3 1.5mL microfuge tubes

6.3.4 Parafilm

6.3.5 Disposable Plastic Luer Lock Syringe – 3mL, 6mL, or 10mL

6.3.6 Nylon or PTFE Syringe Filters, 0.45µm

6.3.7 Weigh paper

6.4 Equipment

6.4.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system

6.4.2 Analytical Balance

6.4.3 Micro Analytical Balance

6.4.4 Ultrasonic Bath

6.4.5 Heated Ultrasonic Bath (Required for Determination of Dihydroberberine.)

6.4.6 Wrist Action Shaker

6.4.7 Stir Plate

6.4.8 Microfuge

6.4.9 10mL Pipette

6.4.10 1mL Pipette

6.4.11 200µL Pipette

7.0 Preparation of Mobile Phase, Diluent, Samples and Standards for Determination of Berberine

7.1 Mobile Phase A – 10mM Ammonium Acetate in H_2O

7.1.1 Transfer ~770.8 mg of ammonium acetate to a 1000-mL mobile phase bottle.

7.1.2 Add 1000 mL of H_2O, and mix to dissolve.

7.2 Mobile Phase B – Acetonitrile

7.3 Diluent– H_2O:ACN (50:50)

7.3.1 Transfer 500 mL of ACN to a 1000-mL mobile phase bottle.

7.3.2 Add 500 mL of H_2O, and mix well.

7.3.3 **Equilibrate to room temperature prior to use.**

7.4 Stock Standard Preparation (250 µg/mL)

7.4.1 Accurately weigh and transfer about 25 mg of reference standard into a 100-mL volumetric flask.

7.4.2 Dilute to volume using Diluent.

7.4.3 Sonicate for 15 minutes or until completely dissolved.

7.5 Working Standard Preparation (10 µg/mL)

7.5.1 Transfer 4.0 mL of the Stock Standard into a 100-mL volumetric flask.

7.5.2 Dilute to volume using Diluent, and mix well.

7.6 Sample Preparation

7.6.1 Specific sample testing details are provided in each product profile. If a specific testing details section is not available, follow preparation procedure as described below, maintaining concentration within the linear range of this method.

7.6.2 The linear range of the method is 0.01 mg/mL – 0.1 mg/mL. All working sample preparations must be within the linear range.

7.6.3 For finished products, pool at least 20 dosage units as required and homogenize using a mortar and pestle.

7.6.4 Based on the label claim and fill or tablet weight for finished products or expected potency for raw materials, weigh an amount greater than the minimum weight of the analytical balance which is sufficient to generate a concentration within the linear range when dissolved in Diluent in an appropriately sized volumetric flask. To handle large volumes, the sample may be initially prepared at a higher concentration and subsequently diluted to within the linear range.

7.6.5 Dilute to the final volume using Diluent, and sonicate for 15 minutes.

7.6.6 Before injection, insoluble matter should be removed via filtration using a nylon syringe filter. Discard at least the first 0.5mL of filtrate before collecting a portion for analysis. Dilute filtrate as needed then add 1mL of the final dilution to an HPLC vial for analysis.

7.6.6.1 Alternatively, samples and standards can also be centrifuged at 6000 RPM for 5 minutes in a microfuge to pellet insoluble matter.

8.0 Preparation of Mobile Phase, Extraction Solvent, Diluent, Samples and Standards for Determination of Dihydroberberine

8.1 Mobile Phase A – 10mM Ammonium Acetate in H_2O

8.1.1 Transfer ~770.8 mg of ammonium acetate to a 1000-mL mobile phase bottle.

8.1.2 Add 1000 mL of H_2O, and mix to dissolve.

8.2 Mobile Phase B – Acetonitrile

8.3 Extraction Solvent – MeOH:DMSO (50:50)

8.3.1 Transfer 500 mL of MeOH to a 1000-mL mobile phase bottle.

8.3.2 Add 500 mL of DMSO, and mix well.

8.3.3 **Equilibrate to room temperature prior to use.**

8.4 Diluent– H_2O:ACN (50:50)

8.4.1 Transfer 500 mL of ACN to a 1000-mL mobile phase bottle.

8.4.2 Add 500 mL of H_2O, and mix well.

8.4.3 **Equilibrate to room temperature prior to use.**

8.5 *Dihydroberberine is extremely unstable in solution and quickly oxidizes to form dynamic mixtures of Berberine / Dihydroberberine. The present method overcomes this complication by quantitatively forcing this conversion (to Berberine) to completion. Both standard and sample preparations are incubated with hydrogen peroxide, enabling Dihydroberberine to be quantified as Berberine.*

8.6 Stock Standard Preparation (100 µg/mL) **(Use Red Glassware.)**

8.6.1 Accurately weigh and transfer ~5 mg of reference standard into a 50-mL volumetric flask.

8.6.2 Add ~30-mL of Extraction Solvent and mechanically shake for 15 minutes.

8.6.3 Fill to volume and sonicate at room temperature for 5 minutes.

8.7 Working Standard Preparation (10 µg/mL)

8.7.1 Using a glass volumetric pipet, transfer 5.0 mL of the Stock Standard into a 50-mL volumetric flask then add 500-µL of H_2O_2. Swirl briefly to mix then seal the flask with parafilm.

8.7.2 Transfer the flask to a heated ultrasonicator (preheated to 40°C) and sonicate for 1 hour. Remove from bath and let cool to room temperature.

8.7.3 Dilute to volume using Diluent, and mix well.

8.8 Sample Preparation **(Use Red Glassware.)**

8.8.1 Specific sample testing details are provided in each product profile. If a specific testing details section is not available, follow preparation procedure as described below, maintaining concentrations as prescribed below.

8.8.2 Pool at least 20 dosage units and homogenize as appropriate (e.g. grind tablets / capsule fill / powders / stick pack contents by mortar and pestle, cryogenically

powder and dissolve gummies, etc.) Transfer sufficient sample (based on the raw material manufacturer assay value / finished product profile) into a 50-mL volumetric flask in order to generate a sample stock that is ~0.1 mg/mL Dihydroberberine. (Do not deviate from this sample stock concentration.) Add ~ 30-mL Extraction Solvent and mechanically shake for 15 minutes then fill to volume and sonicate at room temperature for 5 minutes.

8.8.3 Using a glass volumetric pipet, transfer 5.0 mL of the stock sample into a 50-mL volumetric flask then add 500-µL of H_2O_2. Swirl briefly to mix then seal the flask with parafilm. Transfer the flask to a heated ultrasonicator (preheated to 40°C) and sonicate for 1 hour. Remove from bath and let cool to room temperature. Dilute to volume using Diluent and mix well. Filter a 5mL aliquot for analysis, discarding the first 3-4mL of filtrate.

9.0 Test Conditions

9.1 Gradient

Time	%A	%B
0.00	70	30
8.00	20	80
8.01	70	30
12.00	70	30

9.2 Column – Phenomenex Kinetex, XB-C18, 5um, 100Å, LC column, 250mm x 4.6mm, or equivalent.

9.3 Flow Rate – 1.0mL/min

9.4 UV Detection – 340nm

9.5 3D Spectral Range – 200nm – 500nm

9.6 Injection Volume - 10µL

9.7 Column Temperature – 25°C

9.8 Retention Time – About 9.5 min

9.9 Recommended Sequence

9.9.1 Make at least 2 injections of a Blank (Diluent).

9.9.2 Make five injections of the Working Standard.

9.9.3 Make a single injection of each Sample Preparation.

9.9.4 Make a single injection of the Working Standard after every six samples and at the end of the run.

9.10 System Suitability

9.10.1 The %RSD of five consecutive injections of Working Standard is NMT 5.0%.

9.10.2 The %RSD of all standard injections is NMT 5%.

9.11 Column Wash and Storage

9.11.1 Wash the column with ACN:H_2O (50:50) at 1 mL/min for at least 15 min.

9.11.2 Store the column with ACN:H_2O (50:50).

10.0 Calculations

10.1 Example calculations for determining finished product % label or raw material % purity

10.1.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount in mg (solids) or mL (liquids)

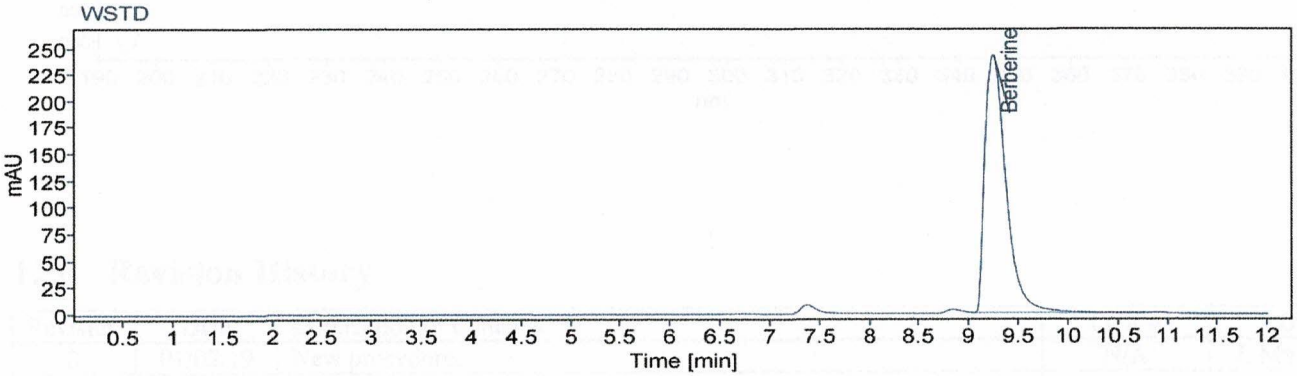
V_{spl} Volume of the sample preparation accounting for dilutions in mL

SS Serving size: Weight of a single dosage unit in mg for tablets and capsules, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

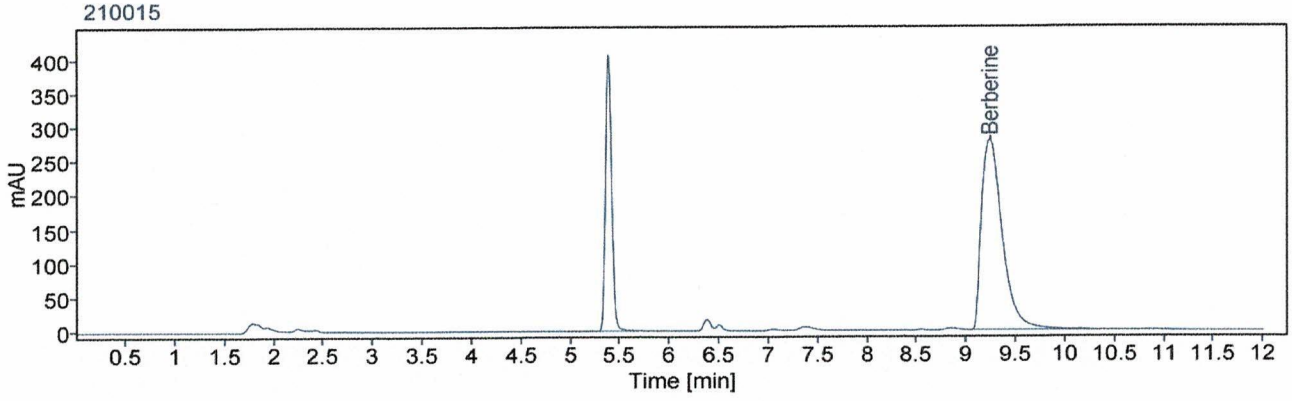
LA Label amount in mg per dose or 1 for raw materials

11.0 Example Chromatography and Spectrum

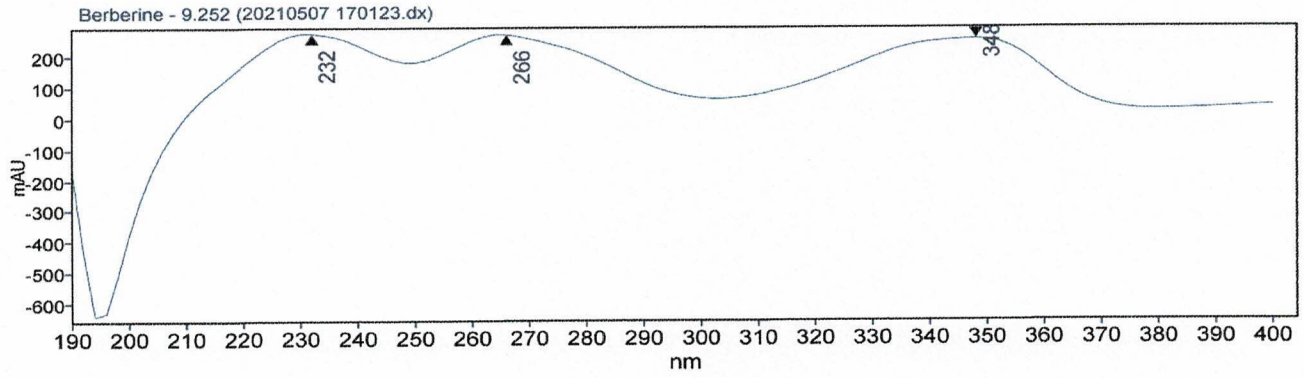
11.1 Working Standard



11.2 Sample



11.3 UV Spectrum of Berberine



12.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/02/19	New procedure.	N/A	J. Maignan
1	08/05/22	Update for consistency with current methods and lab practices, add recommended sequence, add system suitability section, add expected retention time, add linear range, simplify standard preparation.	CC-22-0334	S. Sassman
2	03/26/24	Update for consistency with current methods and lab practices. Added determination of Dihydroberberine.	CC-24-0117	C. Perry